CURRENT PROTOCOLS IN IMMUNOLOGY

VOLUME 1

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Enzyme-Linked Immunosorbent Assays

This unit describes six different ELISA systems for the detection of specific antibodies, soluble antigens, or cell-surface antigens. In all six systems, soluble reactants are removed from solution after specifically binding to solid-phase reactants. Table 2.1.1 summarizes the different ELISA protocols, which are illustrated in Figures 2.1.1-2.1.6.

In the first four protocols, solid-phase reactants are prepared by adsorbing an antigen or antibody onto plastic microtiter plates; in the next two protocols, the solid-phase reactants are cell-associated molecules. In all protocols, the solid-phase reagents are incubated with secondary or tertiary reactants covalently coupled to an enzyme. Unbound conjugates are washed out and a chromogenic or fluorogenic substrate is added.

Table 2.1.1 Summary of ELISA Protocols

ELISA protocol	Uses	Required reagents	Comments
Indirect	Antibody screening; epitope mapping	Antigen, pure or semipure: test solution containing antibody; enzyme conjugate that binds Ig of immunized species	Does not require the use of preexisting specific antibodies; requires relatively large amounts of antigen
Direct competitive	Antigen screening; detect soluble antigen	Antigen, pure or semipure; test solution containing antigen; enzyme-antibody conjugate specific for antigen	Rapid assay with only two steps; excellent for measuring antigenic cross-reactivity
Antibody-sandwich	Antigen screening; detect soluble antigen	Capture antibody (purified or semi-purified specific antibody); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Most sensitive antigen assay; requires relatively large amounts of pure or semi-pure specific antibody (capture antibody)
Double antibody–sandwich	Antibody-screening; epitope mapping	Capture antibody: (specific for Ig of immunized species); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Does not require purified antigen; relatively long assay with five steps
Direct cellular	Screen cells for expression of antigen; measure cellular antigen expression	Cells that express antigen of interest; enzyme-antibody conjugate specific for cellular antigen	Sensitive assay for bulk screening; insensitive to heterogeneity of expression in mixed population of cells
Indirect cellular	Screen for antibodies against cellular antigens	Cells used for immunizing; test solution containing antibodies; enzyme conjugate that binds Ig of immunized species	May not detect antibodies specific for cellular antigens expressed at a low density

As the substrate is hydrolyzed by the bound enzyme conjugate, a colored or fluorescent product is generated. Finally, the product is detected visually or with a microtiter plate reader. The amount of product generated is proportional to the amount of analysate in the test mixture. The first support protocol can be used to optimize the different ELISAs. The second support protocol presents a method for preparing alkaline phosphatase conjugates.

INDIRECT ELISA TO DETECT SPECIFIC ANTIBODIES

BASIC PROTOCOL

This assay is useful for screening antisera or hybridoma supernatants for specific antibodies when milligram quantities of purified or semi-purified antigen are available (1 mg of purified antigen will permit screening of 80 to 800 microtiter plates; Fig. 2.1.1). Antibodies are detected by coating the wells of microtiter plates with antigen, incubating the coated plates with test solutions containing specific antibodies, and washing away unbound antibodies. A solution containing a developing reagent, (e.g., alkaline phosphatase conjugated to protein A, protein G, or antibodies against the test solution antibodies) is then added to the plate. After incubation, unbound conjugate is washed away and substrate solution is added. After a second incubation, the amount of substrate hydrolyzed is assessed with a spectrophotometer or spectrofluorometer. The measured amount is proportional to the amount of specific antibody in the test solution. Visual inspection can also be used to detect hydrolysis.

Materials

Developing reagent: protein A–alkaline phosphatase conjugate (Sigma #P9650), protein G–alkaline phosphatase conjugate (Calbiochem #539304), *or* anti-Ig-alkaline phosphatase conjugate (second support protocol)
Antigen solution
PBS (*APPENDIX 2*) containing 0.05% NaN_3 (PBSN)
Water, deionized or distilled
Blocking buffer
Test antibody samples
4-methylumbelliferyl phosphate (MUP) *or* *p*-nitrophenyl phosphate (NPP) substrate solution
0.5 M NaOH (optional)
Multichannel pipet and disposable pipet tips
Immulon 2 (Dynatech #011-010-3450), Immulon 4 (Dynatech #011-010-3850), or equivalent microtiter plates
Plastic squirt bottles
Microtiter plate reader (optional)—spectrophotometer with 405-nm filter or spectrofluorometer (Dynatech #011-970-1900) with 365-nm excitation filter and 450-nm emission filter

Determine developing reagent and antigen concentrations

1. Determine the optimal concentration of the developing reagent (conjugate) by criss-cross serial-dilution analysis (see first support protocol).

   Good conjugates of many specificities are available commercially. Choice of developing reagent (i.e., conjugate) is determined by the goals of the assay. If it is necessary to detect all antibodies that bind to antigen, conjugates prepared with antibodies specific for Ig κ and λ light chains should be used. Alternatively, protein A– or protein G–enzyme conjugates may be preferable when screening monoclonal antibodies. Specific monoclonal antibodies that bind protein A or protein G are easy to purify and characterize.

2. Determine the final concentration of antigen coating reagent by criss-cross serial-dilution analysis (see first support protocol). Prepare an antigen solution in PBSN at this final concentration. The final concentration of antigen is usually 0.2 to 10.0 μg/ml. Prepare ~6 ml antigen solution for each plate.

Pure antigen solution concentrations are usually ≤2 μg/ml. Although pure antigen preparations are not essential, >3% of the protein in the antigen solution should be the antigen. The total concentration of protein in the antigen solution should be increased for semipurified antigen preparations. Do not raise the total protein concentration in the antigen solution to >10 μg/ml, since this concentration usually saturates >85% of the available sites on Immulon microtiter plates. For some antigens, coating may occur more efficiently at different pHs.

Coat plate with antigen

3. Using a multichannel pipet and tips, dispense 50 μl antigen solution into each well of an Immulon microtiter plate. Tap or shake the plate to ensure that the antigen solution is evenly distributed over the bottom of each well.

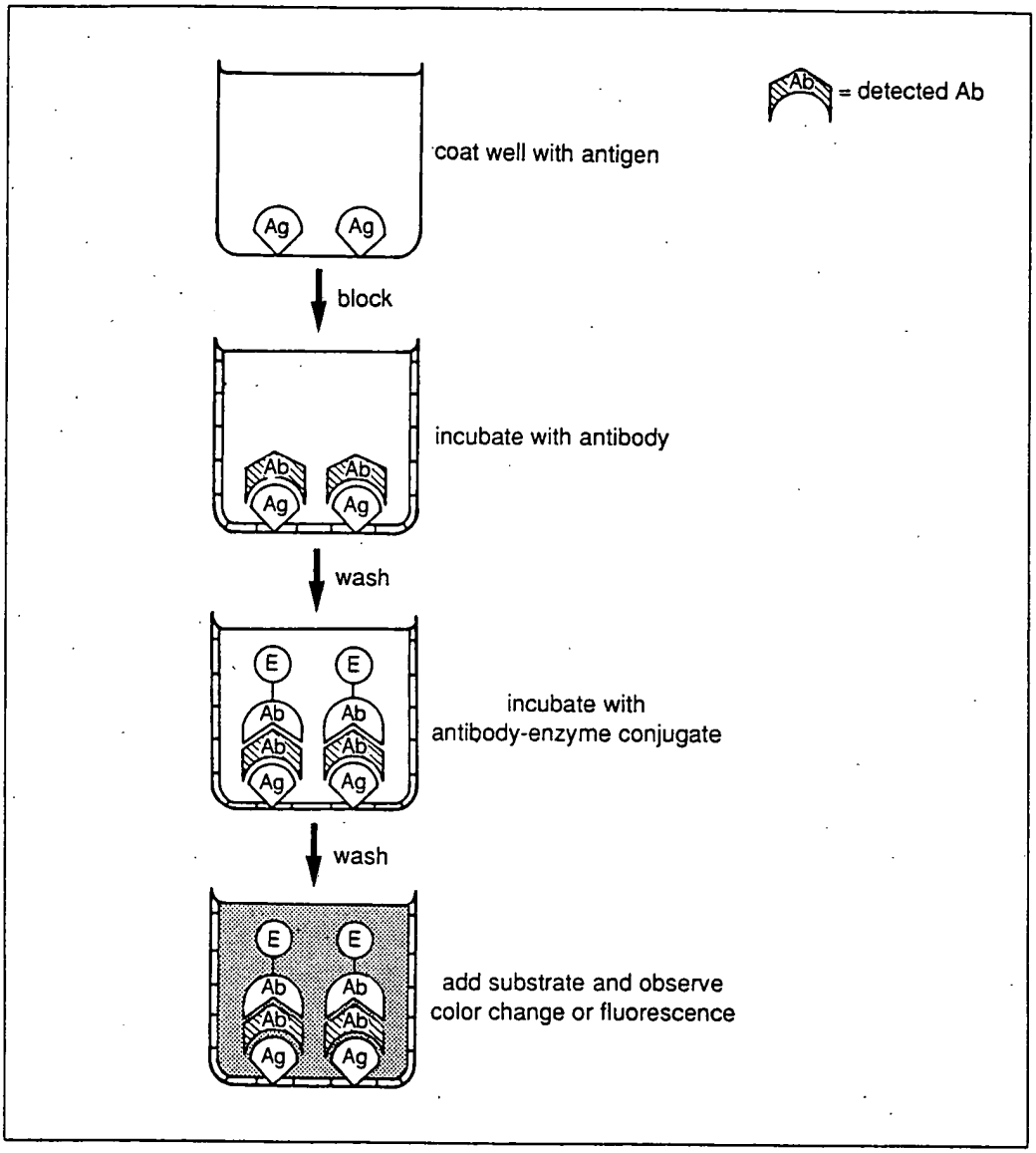

Figure 2.1.1 Indirect ELISA to detect specific antibodies. Ag = antigen; Ab = antibody; E = enzyme.

4. Wrap coated plates in plastic wrap to seal and incubate overnight at room temperature or 2 hr at 37°C.

   Individual adhesive plate sealers are sold commercially but plastic wrap is easier to use and works as well. Sealed plates can be stored at 4°C with antigen solution for months.

5. Rinse coated plate over a sink by filling wells with deionized or distilled water dispensed either from a plastic squirt bottle or from the tap. Flick the water into the sink and rinse with water two more times, flicking the water into the sink after each rinse.

Block residual binding capacity of plate

6. Fill each well with blocking buffer dispensed as a stream from a squirt bottle and incubate 30 min at room temperature.

   Residual binding capacity of the plate is blocked in this step. Tween 20 (0.05%) by itself is more effective at blocking than any protein tested, but because the combination of protein and Tween 20 may be more effective than Tween 20 alone in some cases, bovine serum albumin (BSA; 0.25%) is included in the blocking buffer.

7. Rinse plate three times in water as in step 5. After the last rinse, remove residual liquid by wrapping each plate in a large paper tissue and gently flicking it face down onto several paper towels laying on the benchtop.

   Rinsing with water is cheaper and easier than rinsing with buffered solutions and is as effective.

Add antibody to plate

8. Add 50 µl antibody samples diluted in blocking buffer to each of the coated wells, wrap plate in plastic wrap, and incubate ≥2 hr at room temperature.

   While enough antibody may be bound after 1 to 2 hr to generate a strong signal, equilibrium binding is generally achieved after 5 to 10 hr. Thus, the specific signal may be significantly increased by longer incubations.

   For this and all steps involving the delivery of aliquots of many different solutions to microtiter plates with multichannel pipets, such as the primary screening of hybridoma supernatants, the same pipet tips can be reused for hundreds of separate transfers. Wash tips between transfers by expelling any liquid remaining in the tips onto an absorbent surface of paper tissues, rinsing tips five times in blocking buffer, and carefully expelling any residual liquid from tips onto the tissues. Avoid bubbles in the tips; any tip with intractable bubbles should be replaced.

Wash the plate

9. Rinse plate three times in water as in step 5.

10. Fill each well with blocking buffer, vortex, and incubate 10 min at room temperature.

    Plates are vortexed to remove any reagent remaining in the corners of the wells.

11. Rinse three times in water as in step 5. After the final rinse, remove residual liquid as in step 7.

Add developing reagent to plate

12. Add 50 µl developing reagent in blocking buffer (at optimal concentration determined in step 1) to each well, wrap in plastic wrap, and incubate ≥2 hr at room temperature.

    The strength of the signal may be increased by longer incubations (see annotation to step 8).

13. Wash plates as in steps 9 to 11.

After final rinsing, plates may be wrapped in plastic wrap and stored for months at 4°C prior to adding substrate.

Add substrate and measure hydrolysis

14. Add 75 µl MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

15. Monitor hydrolysis qualitatively by visual inspection or quantitatively with a microtiter plate reader (see below). Hydrolysis can be stopped by adding 25 µl of 0.5 M NaOH.

    a. Visually, hydrolysis of NPP can be detected by the appearance of a yellow color. If using a microtiter plate reader to measure NPP hydrolysis, use a 405-nm filter.

    b. Visually, hydrolysis of MUP can be monitored in a darkened room by illumination with a long-wavelength UV lamp. If using a microtiter plate spectrofluorometer to measure MUP hydrolysis, use a 365-nm excitation filter and a 450-nm emission filter.

The fluorogenic system using the MUP substrate is 10 to 100 times faster than the chromogenic system using NPP. Furthermore, the rate of spontaneous hydrolysis of MUP is much lower than that of NPP.

To detect bound antibodies that are present at low concentration, measure hydrolysis at a later time. To calculate when to measure hydrolysis the second time, remember that the amount of hydrolysis is almost directly proportional to the time of hydrolysis. For example, if the hydrolysis in the wells of interest reads 200 at 1 hr and a reading of 2000 is desired, incubate the plate ~10 hr before taking the second reading.

ALTERNATE PROTOCOL

DIRECT COMPETITIVE ELISA TO DETECT SOLUBLE ANTIGENS

This assay is used to detect or quantitate soluble antigens and is most useful when both a specific antibody and milligram quantities of purified or semi-purified antigen are available (Fig. 2.1.2). To detect soluble antigens, plates are coated with antigen and the binding of specific antibody-enzyme conjugates to antigen-coated plates is inhibited by test solutions containing soluble antigen. After incubation with mixtures of the conjugate and inhibitor in antigen-coated wells, unbound conjugate is washed away and substrate is added. The amount of antigen in the test solutions is proportional to the inhibition of substrate hydrolysis and can be quantitated by interpolation onto an inhibition curve generated with serial dilutions of a standard antigen solution.

The direct assay may also be adapted as an indirect assay by substituting specific antibody for specific antibody-enzyme conjugate. The amount of specific antibody bound is then detected using a species-specific or isotype-specific conjugate as a tertiary reactant.

Additional Materials

Specific antibody–alkaline phosphatase conjugate (second support protocol)
Standard antigen solution
Test antigen solutions
Round- or cone-bottom microtiter plates

1. Determine the optimal concentration of coating reagent and antibody–alkaline phosphatase conjugate by criss-cross serial-dilution analysis in which the concentrations of both the antigen (coating reagent) and the conjugate (developing reagent) are

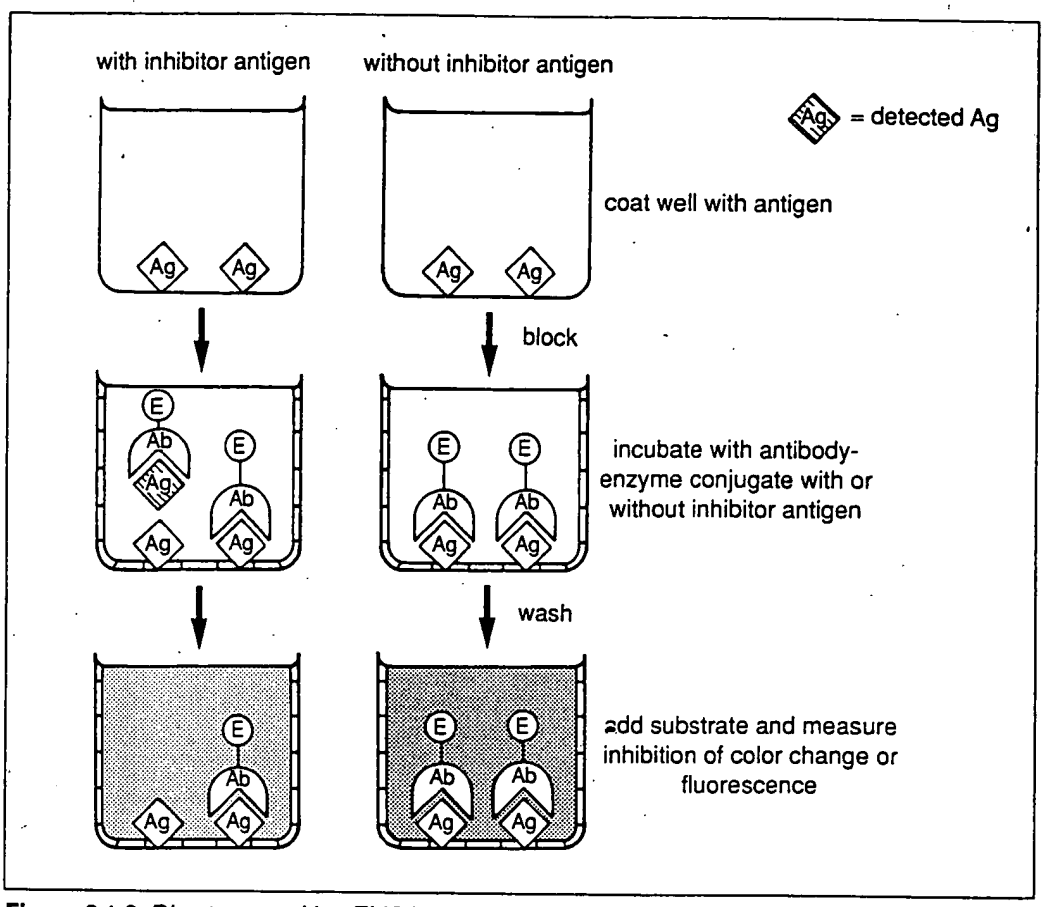

Figure 2.1.2 Direct competitive ELISA to detect soluble antigens. Ag = antigen; Ab = antibody; E = enzyme.

varied (see first support protocol). Prepare a 2× conjugate solution by diluting the specific antibody–alkaline phosphatase conjugate in blocking buffer to twice the optimal concentration.

The final concentration is usually 25 to 500 ng antibody/ml. Prepare 3 ml antibody–alkaline phosphatase conjugate for each plate.

2. Coat and block wells of an Immulon microtiter plate with 50 µl antigen solution as in steps 2 to 7 of the basic protocol.

3. Prepare six 1:3 serial dilutions of standard antigen solution in blocking buffer (see first support protocol for preparation of serial dilutions)—these antigen concentrations will be used in preparing a standard inhibition curve (see step 10).

Antigen concentrations should span the dynamic range of inhibition. The dynamic range of inhibition is defined as that range of inhibitor concentrations wherein changes of inhibitor concentration produce detectable changes in the amount of inhibition. The dynamic range of inhibition is empirically determined in an initial assay in which antigen concentration is typically varied from the micromolar (10^{-6} M) to the picomolar (10^{-12} M) range. For most protein antigens, initial concentration should be ~10 µg/ml, followed by nine 1:4 serial dilutions in blocking buffer. These antigen dilutions are assayed for their ability to inhibit the binding of conjugate to antigen-coated plates under standard assay conditions. From this initial assay, six 1:3 antigen dilutions spanning the dynamic range of inhibition are selected for further use as standard antigen-inhibitor dilutions. Prepare ≥75 µl of each dilution for each plate to be assayed.

Inhibitor curves are most sensitive in the region of the curve where small changes in inhibitor concentrations produce maximal changes in the amount of inhibition. This

region of the curve normally spans 15% to 85% inhibition. In most systems, this range of inhibition is produced by concentrations of inhibitor between 1 and 250 ng/ml.

4. Mix and incubate conjugate and inhibitor by adding 75 µl of 2× conjugate solution (from step 1) to each well of a round- or cone-bottom microtiter plate, followed by 75 µl inhibitor—either test antigen solution or standard antigen solution (from step 3). Mix the conjugate and inhibitor solutions by pipetting up and down in the pipet tip three times (see annotation to step 8 in the basic protocol) and incubate ≥30 min at room temperature.

   For accurate quantitation of the amount of antigen in the test solutions, test antigen solutions should inhibit conjugate binding between 15% to 85%. It may be necessary to assay two or three different dilutions of the test solutions to produce inhibitions within this range.

5. Prepare uninhibited control samples by mixing equal volumes of 2× conjugate solution and blocking buffer.

6. Transfer 50 µl of the mixture of conjugate plus inhibitor (from step 4) or conjugate plus blocking buffer (from step 5) to an antigen-coated plate (from step 2) and incubate 2 hr at room temperature.

   If samples are to be assayed in duplicate, the duplicates should be in adjacent columns on the same plate. Reserve column 11 for uninhibited control samples (step 5) and column 12 for substrate alone without any conjugate. If the concentration of antigen in the test samples is to be accurately quantitated, dilutions of homologous antigen solutions (step 3) should be included on each plate.

7. Wash plate as in steps 9 to 11 of the basic protocol.

8. Add 75 µl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

9. Read plates on the microtiter plate reader after ≥1 hr, at which time enough substrate has been hydrolyzed in the uninhibited reactions to permit accurate measurement of the inhibition.

10. Prepare a standard antigen-inhibition curve constructed from the inhibitions produced by the dilutions of the standard antigen solutions from step 3. Plot antigen concentration on the x axis, which is a log scale, and fluorescence or absorbance on the y axis, which is a linear scale.

11. Interpolate the concentration of antigen in the test solutions from the standard antigen-inhibition curve.

    The dynamic range of the inhibition curve may deviate from linearity if the specific antibodies are heterogeneous and possess significantly different affinities or if the standard antigen preparation contains heterogeneous forms of the antigen. Antigen concentration in test samples can be accurately interpolated from the inhibition curve as long as the test antigen is antigenically identical to the standard antigen and the concentration of test antigen falls within the dynamic range of inhibition.

ANTIBODY-SANDWICH ELISA TO DETECT SOLUBLE ANTIGENS

ALTERNATE PROTOCOL

Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid phase (Fig. 2.1.3). To detect antigen, the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

Additional Materials

Specific antibody or immunoglobulin fraction from antiserum or ascites fluid, *or* hybridoma supernatant (*UNIT 2.6*)

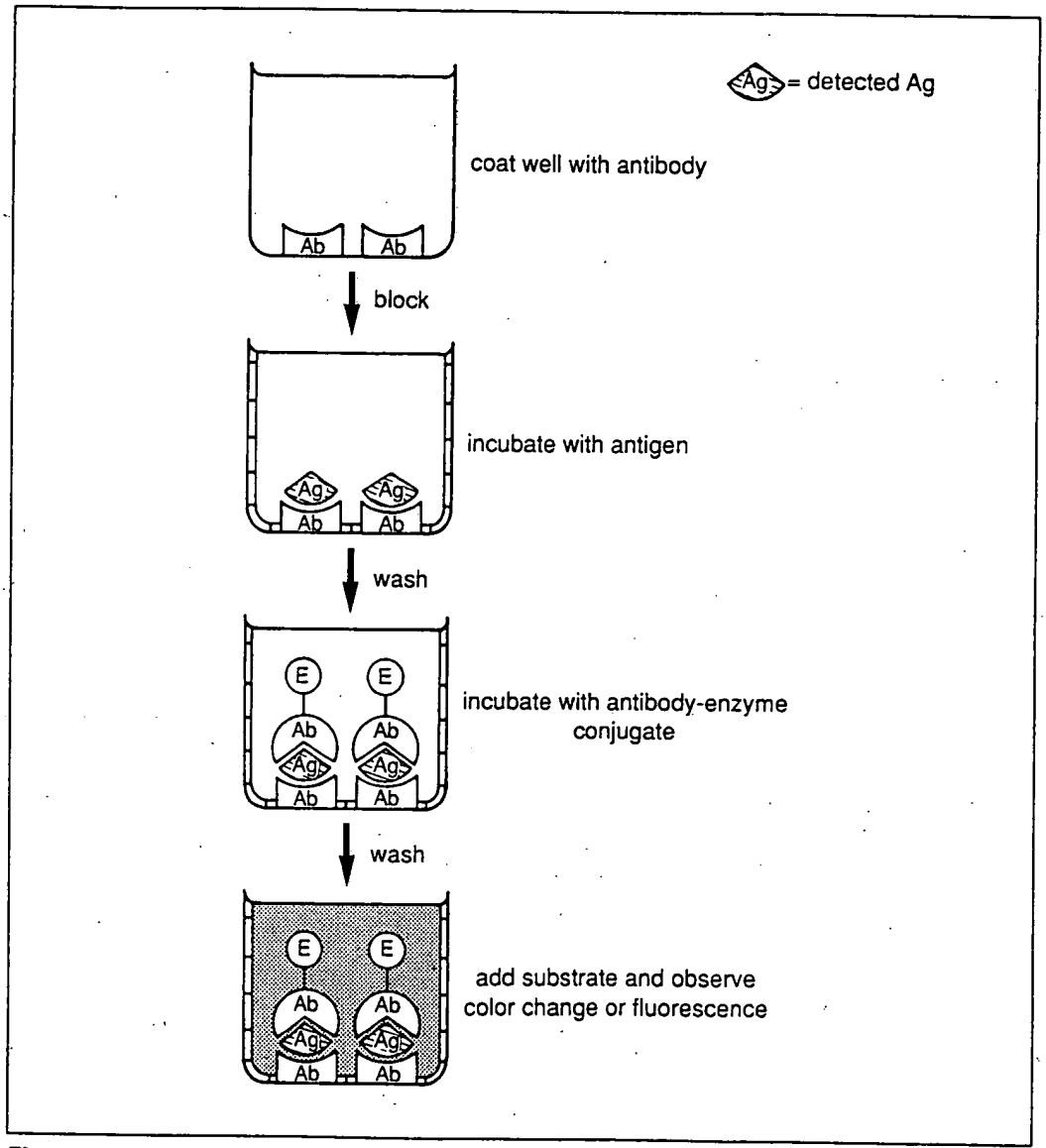

Figure 2.1.3 Antibody-sandwich ELISA to detect antigen. Ag = antigen; Ab = antibody; E = nzyme.

1. Prepare the capture antibody by diluting specific antibody or immunoglobulin fraction in PBSN to a final concentration of 0.2 to 10 μg/ml.

   The capture antibodies can be monoclonal or polyclonal.

   If the immunoglobulin fraction from an antiserum or ascites fluid is used, the concentration of total protein may need to be increased to compensate for the lower content of specific antibody. Little advantage is gained by increasing the total protein concentration in the capture antibody solution beyond 10 μg/ml.

2. Determine the concentration of capture antibody and conjugate necessary to detect the desired concentration of antigen by criss-cross serial-dilution analysis (see first support protocol). Prepare a capture antibody solution in PBSN at this concentration.

3. Coat wells of an Immulon plate with capture-antibody solution as in steps 3 to 5 of the basic protocol.

4. Block wells as in steps 6 and 7 of the basic protocol.

5. Prepare a standard antigen-dilution series by successive 1:3 dilutions of the homologous antigen stock in blocking buffer (see first support protocol).

   In order to measure the amount of antigen in a test sample, the standard antigen-dilution series needs to span most of the dynamic range of binding. This range typically spans from 0.1 to 1000 ng antigen/ml. The dynamic range of binding is defined as that range of antigen concentrations wherein small, incremental changes in antigen concentration produce detectable differences in the amount of antigen bound (see annotation to step 3, in the preceding alternate protocol). In most assay systems, the amount of antigen in a test solution is most accurately interpolated from the standard curve if it produces between 15% to 85% of maximal binding.

   Note: *While standard curves are necessary to accurately measure the amount of antigen in test samples, they are unnecessary for qualitative "yes/no" answers.*

6. Prepare dilutions of test antigen solutions in blocking buffer.

   It may be necessary to assay one or two serial dilutions of the initial antigen test solution to ensure that at least one of the dilutions can be accurately measured. For most assay systems, test solutions containing 1 to 100 ng/ml of antigen can be accurately measured.

7. Add 50-μl aliquots of the antigen test solutions and the standard antigen dilutions (from step 5) to the antibody-coated wells and incubate ≥2 hr at room temperature.

   For accurate quantitation, samples should be run in duplicate or triplicate, and the standard antigen-dilution series should be included on each plate (see step 5). Pipetting should be performed rapidly to minimize differences in time of incubation between samples.

8. Wash plate as in steps 9 to 11 of the basic protocol.

9. Add 50 μl specific antibody–alkaline phosphatase conjugate and incubate 2 hr at room temperature.

   The conjugate concentration is typically 25 to 400 ng specific antibody/ml.

   When the capture antibody is specific for a single determinant, the conjugate must be prepared from antibodies which recognize different determinants that remain available after the antigen is bound to the plate by the capture antibody.

10. Wash plate as in steps 9 to 11 of the basic protocol.

11. Add 75 μl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

12. Read the plate on a microtiter plate reader.

    To quantitate low-level reactions, the plate can be read again after several hours of hydrolysis.

13. Prepare a standard curve constructed from the data produced by serial dilutions of the standard antigen (step 5). Plot antigen concentration on the *x* axis which is a log scale, and fluorescence or absorbance on the *y* axis which is a linear scale.

14. Interpolate the concentration of antigen in the test solutions from the standard curve.

DOUBLE ANTIBODY–SANDWICH ELISA TO DETECT SPECIFIC ANTIBODIES

ALTERNATE PROTOCOL

This assay is especially useful when screening for specific antibodies in cases when a small amount of specific antibody is available and purified antigen is unavailable (Fig. 2.1.4). Additionally, this method can be used for epitope mapping of different monoclonal antibodies that are directed against the same antigen. Plates are coated with capture antibodies specific for immunoglobulin from the immunized species. The test antibody solution is incubated on the plates coated with the capture antibodies. Plates are then washed, incubated with antigen, washed again, and incubated with specific antibody conjugated to an enzyme. After incubation, unbound conjugate is washed out and substrate is added. Wells that are positive for hydrolysis may contain antibodies specific for the antigen.

Additional Materials

Capture antibodies specific for immunoglobulin from the immunized species
Specific antibody–alkaline phosphatase conjugate

1. Coat wells of an Immulon microtiter plate with 50 µl of 2 to 10 µg/ml capture antibodies as in steps 2 to 5 of the basic protocol.

   NOTE: *Capture antibodies must not bind the antigen or conjugate antibodies. When analyzing hybridoma supernatants or ascites fluid, coat plates with 2 µg/ml capture antibody. When analyzing antisera, coat plates with 10 µg/ml capture antibody.*

2. Block wells as in steps 6 and 7 of the basic protocol.

3. Prepare dilutions of test antibody solutions in blocking buffer. Add 50 µl to coated wells and incubate ≥2 hr at room temperature.

   Hybridoma supernatants, antisera, or ascites fluid can be used as the test samples. Dilute hybridoma supernatants 1:5 and antisera or ascites fluid 1:200.

4. Wash plate as in steps 9 to 11 of the basic protocol.

5. Prepare an antigen solution in blocking buffer containing 20 to 200 ng/ml antigen.

   Although purified antigen preparations are not essential, the limit of detectability for most protein antigens in this type of system is 2 to 20 ng/ml. A concentration of 20 to 200 ng antigen/ml is recommended.

6. Add 50-µl aliquots of the antigen solution to antibody-coated wells and incubate ≥2 hr at room temperature.

7. Wash plate as in steps 9 to 11 of the basic protocol.

8. Add 50 µl specific antibody–alkaline phosphatase conjugate to the wells and incubate 2 hr at room temperature.

The conjugate antibodies must not react with the capture antibody or the test antibody. The conjugate concentration is typically between 25 to 500 ng specific antibody/ml, and should be high enough to result in ~0.50 absorbance units/hr at 405 nm when using NPP as a substrate or a signal of 1000 to 1500 fluorescence units/hr when using MUP as a substrate. If no specific antibodies from the appropriate species

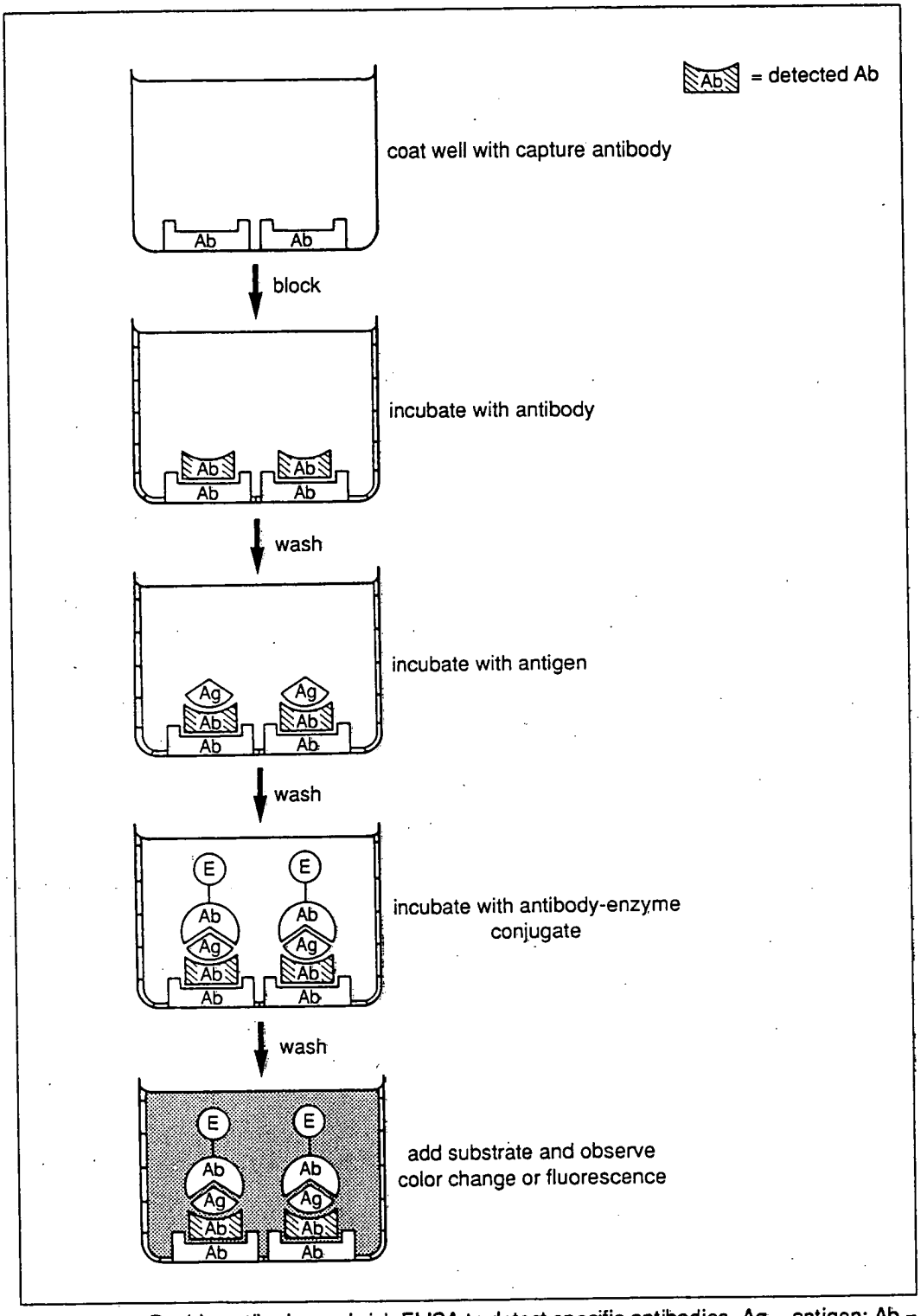

Figure 2.1.4 Double antibody-sandwich ELISA to detect specific antibodies. Ag = antigen; Ab = antibody; E = enzyme.

are available to serve as a positive control, then a positive control system should be constructed out of available reagents. Such reagents can be found in Linscott's Directory of Immunological and Biological Reagents.

9. Wash plate as in steps 9 to 11 of the basic protocol.

10. Add 75 µl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature. After 1 hr, examine hydrolysis visually or spectrophotometrically (see step 15 of the basic protocol).

    In order to detect low-level reactions, the plate can be read again after several hours or days of hydrolysis.

11. Check for false positives by rescreening samples that test positive for antigen-specific antibody. For each positive sample, coat four wells with capture antibody and arm the capture antibody with test antibody (steps 1 to 4). Incubate two of the wells with antigen (steps 5 to 7) and two of the wells with blocking buffer. Add conjugate and substrate to all four wells (steps 8 to 10) and measure hydrolysis after 1 hr.

    This procedure will eliminate false positives resulting from test antibodies that react with the enzyme-antibody complex.

DIRECT CELLULAR ELISA TO DETECT CELL-SURFACE ANTIGENS

ALTERNATE PROTOCOL

The expression of cell-surface antigens or receptors is measured using existing antibodies or other ligands specific for cell-surface molecules (Fig. 2.1.5). Cells are incubated with enzyme conjugated to antibodies that are specific for a cell-surface molecule. Unbound conjugate is washed away and substrate is added. The level of antigen expression is proportional to the amount of substrate hydrolysis. This procedure can be as sensitive as flow cytometry analysis in quantitating the level of antigen expression on a population of cells (*UNITS 5.1 - 5.4*). Unlike the flow cytometry analysis, however, this method is not sensitive for mixed populations. This assay can be converted to an indirect assay by substituting biotinylated antibody for the enzyme-antibody conjugate, followed by a second incubation with avidin–alkaline phosphatase.

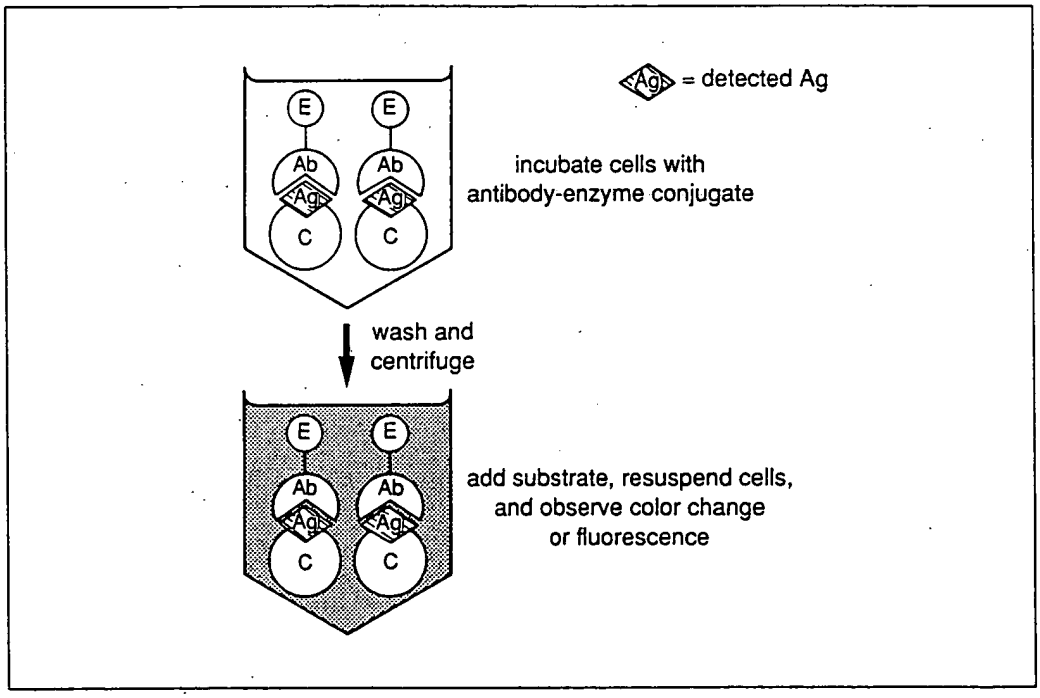

Figure 2.1.5 Direct cellular ELISA to detect cell-surface antigens. Ab = antibody; E = enzyme; C = cell.

Additional Materials

Cell samples
Specific antibody–alkaline phosphatase conjugate (see second support protocol)
Wash buffer, ice-cold

Cone- or round-bottom microtiter plates
Sorvall H-1000B rotor (or equivalent)

1. Determine the optimal number of cells per well and the antibody-conjugate concentration by criss-cross serial-dilution analysis (see first support protocol) using variable numbers of positive- and negative-control cell samples and varying concentrations of antibody-biotin conjugate.

   Titrate cells initially at 1-5 × 10^5/well and conjugate at 0.5 to 10 μg/ml. For preparation and handling of cells, consult steps 2 to 5.

   Because eukaryotic cells express variable amounts of alkaline phosphatase, test cells must be assayed in a preliminary experiment for alkaline phosphatase by incubation with substrate alone. If the test cells express unacceptable levels of alkaline phosphatase, another enzyme conjugate such as β-galactosidase should be used. Both chromogenic and fluorogenic substrates are available for β-galactosidase.

2. Centrifuge cell samples in a table-top centrifuge 5 min in Sorvall H-1000B rotor at 1500 rpm (450 × *g*), 4°C, in a 15- to 50-ml centrifuge tube. Count cells (*APPENDIX 3*) and resuspend in ice-cold wash buffer at 1-5 × 10^6 cells/ml.

   If the surface antigen retains its antigenicity after fixation, cells may be fixed at the beginning of the experiment—but do not fix cells unless it can be demonstrated that the antigenicity is retained after fixation. *Fix cells by suspending in glutaraldehyde (0.5% final; from a 25% stock, EM grade Sigma #G5882), and incubating 30 min at room temperature. Pellet cells, resuspend in PBSLE (see second support protocol), and incubate for 30 min at 37°C. Wash twice in PBSLE and resuspend in wash buffer. Cells can be kept for months at 4°C after fixation.*

3. Dispense 100 μl of cell suspension (1-5 × 10^5 cells) into wells of cone- or round-bottom microtiter plates, and centrifuge 1 min at 450 × *g*, 4°C. Remove supernatant by vacuum aspiration, and disrupt pellet by briefly shaking microtiter plate on a vortex mixer or microtiter plate shaker.

4. Resuspend pellet in 100 μl of conjugate in ice-cold wash buffer at the optimal concentration (see step 1). Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

   Be careful not to splash cell suspensions out of wells.

5. Centrifuge cells 1 min at 450 × *g*, 4°C, remove supernatant by vacuum aspiration, briefly vortex pellet, and resuspend in 200 μl ice-cold wash buffer. Repeat three times.

6. Add 100 μl MUP or NPP substrate solution. Incubate 1 hr at room temperature, resuspending cells by gently shaking at 15-min intervals during hydrolysis.

7. Determine extent of hydrolysis by visual inspection or using a microtiter plate reader.

INDIRECT CELLULAR ELISA TO DETECT ANTIBODIES SPECIFIC FOR SURFACE ANTIGENS

ALTERNATE PROTOCOL

This assay is designed to screen for antibodies specific for cell-surface antigens (Fig. 2.1.6). Antibodies against surface antigens are detected by incubating whole cells with a test solution containing the primary antibody. The unbound antibody is washed away, and the cells are then incubated with an enzyme conjugated to antibodies specific for the primary antibody. Unbound enzyme conjugate is washed away and substrate solution added. The level of bound primary antibody is proportional to the amount of substrate hydrolysis.

Additional Materials

Positive-control antibodies (i.e., those that react with the experimental cells and are from the immunized species)
Negative-control antibodies (i.e., those that do not react with the experimental cells)
Test antibody solution
Antibody– or $F(ab')_2$ (against immunoglobulin from the immunized species)–alkaline phosphatase conjugate
Cone- or round-bottom microtiter plates

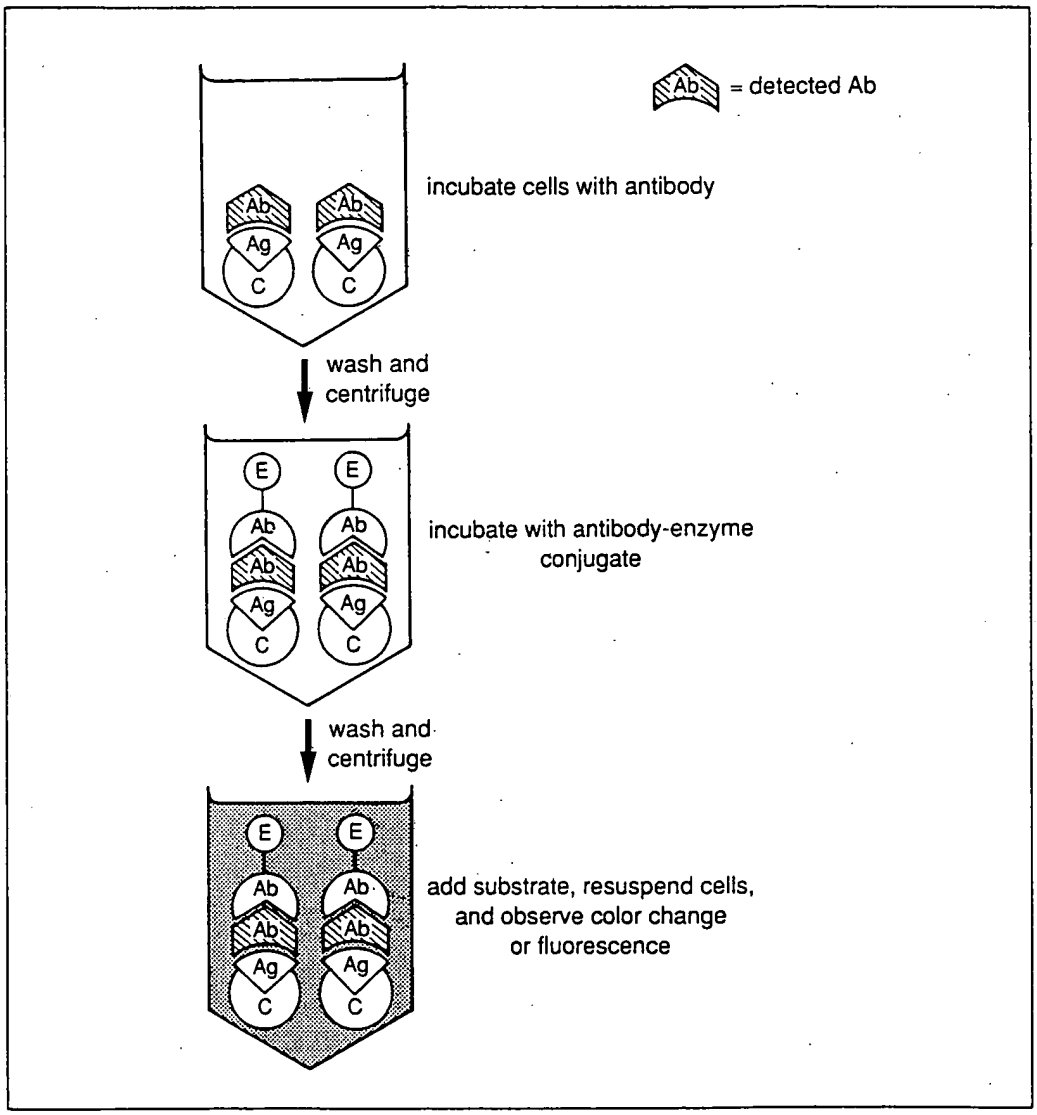

Figure 2.1.6 Indirect cellular ELISA to detect antibodies specific for surface antigens. Ab = antibody; E = enzyme; C = cell.

1. Centrifuge and resuspend cell samples as in step 2 of the previous alternate protocol at 1-5 × 10^6 cells/ml.

   Because this technique detects antibodies against uncharacterized epitopes, fixation prior to analysis is not recommended. Fixation may destroy the antigenicity of the epitope. All steps must be performed at 4°C in physiological buffers containing NaN_3.

   Because eukaryotic cells express variable amounts of alkaline phosphatase, test cells must be assayed for alkaline phosphatase activity. If the endogenous alkaline phosphatase level is too high, another enzyme should be substituted for alkaline phosphatase in the antibody-enzyme conjugate (see annotation to step 1 of the previous alternate protocol).

2. In preliminary assays, determine the optimal number of cells per well and conjugate concentration by criss-cross serial-dilution analysis using positive- and negative-control antibodies instead of test antibodies (see first support protocol). In adapting the criss-cross serial-dilution analysis, whole cells replace the solid-phase coating reagent; see techniques for handling cells are outlined in steps 3 to 8. Set up titrations by varying the number of cells between 1 × 10^5 and 5 × 10^5/well, the concentration of positive- and negative-control antibodies between 0.1 and 10 μg/ml, and the concentration of antibody-enzyme conjugate between 0.1 and 10 μg/ml.

3. Dispense 100 μl of cell suspension (1-5 × 10^5 cells) into wells of round- or cone-bottom microtiter plates. Centrifuge 1 min at 1500 rpm, 4°C, remove supernatant by vacuum aspiration, and disrupt pellet by briefly shaking microtiter plate on the vortex mixer.

4. Resuspend cells in 100 μl solutions containing 1 to 10 μg/ml test antibody or control antibodies in ice-cold wash buffer. Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

   Be careful not to splash cell suspensions out of wells.

5. Centrifuge cells 1 min at 1500 rpm, 4°C, remove supernatant by vacuum aspiration, briefly vortex pellet, and resuspend in 200 μl ice-cold wash buffer. Repeat twice.

6. Resuspend pellet in 100 μl enzyme-antibody conjugate or $F(ab')_2$-enzyme conjugate diluted in ice-cold wash buffer. The optimal concentration of antibody, determined in step 2, is usually 100 to 500 ng/ml. Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

   When working with cells that may express Fc receptors, it is best to use enzyme conjugated to $F(ab')_2$ fragments. $F(ab')_2$ fragments have had the Fc portion of the antibody enzymatically removed and no longer bind to Fc receptors.

7. Wash cells as in step 5. Repeat three times.

8. Add 100 μl MUP or NPP substrate solution. Allow hydrolysis to proceed until the signal has reached the desired levels; resuspend cells by gently shaking at 15 min intervals during hydrolysis. If desired, stop hydrolysis by adding 25 μl of 0.5 M NaOH.

9. Determine extent of hydrolysis by visual inspection or spectrophotometrically using a microtiter plate reader.

CRISS-CROSS SERIAL-DILUTION ANALYSIS TO DETERMINE OPTIMAL REAGENT CONCENTRATIONS

SUPPORT PROTOCOL

Serial dilution titration analyses are performed to determine optimal concentrations of reagents to be used in ELISAs. In this protocol, all three reactants in a three-step ELISA—a primary solid-phase coating reagent, a secondary reagent that binds the primary reagent, and an enzyme-conjugated tertiary developing reagent that binds to the secondary reagent—are serially diluted and analyzed by a criss-cross matrix analysis (Fig 2.1.7). Once the optimal concentrations of reagents to be used under particular assay conditions are determined, these variables are kept constant from experiment to experiment. The coating (primary), secondary, and developing (tertiary) reagents will vary depending upon which of the previous protocols needs to be optimized.

Additional Materials

Coating reagent
Secondary reagent
Developing reagent
17 × 100–mm and 12 × 74–mm test tubes

Secondary reactant

Tertiary reactant (antibody–alkaline phosphatase) (ng/ml)	homologous (antigen) 200	50	12.5	3.12	0.78	0	heterologous (antigen) 200	50	12.5	3.12	0.78	0	Rows
													A
500	over	over	over	3200	1000	0	500	120	40	20	10		B
250	over	over	over	2060	560	0	300	80	20	0	0		C
125	over	over	3650	1370	360	0	195	40	10	10	0		D
62.5	3600	4000	2270	790	240	0	120	30	10	10	10		E
31.25	2700	2100	1200	410	120	0	60	10	10	10	0		F
0	0	0	0	0	0	0	0	0	0	0	0		G
													H
	1	2	3	4	5	6	7	8	9	10	11	12	

Columns

Figure 2.1.7 Results of a criss-cross serial-dilution analysis (for optimization of secondary and tertiary reactant concentrations) of an antibody-sandwich ELISA to detect antigen. The numbers in columns 1 to 11 and rows B to G represent relative fluorescence units observed for each well on a 96-well microtiter plate.

Plates were coated overnight with the capture antibody at 2 µg/ml. The secondary reactants, 4-fold serial dilutions of the homologous antigen and a non-cross-reactive heterologous antigen, were incubated on the plate 2 hr. The tertiary reactant, 2-fold serial dilutions of specific antibody–alkaline phosphatase conjugates, were incubated on th plat 2 hr. After 1 hr of incubation with the substrate MUP, the fluorescence was read in a microtiter plate spectrofluorometer.

Reagent concentrations depend upon individual assay variables that are set by the investigator. If the time of hydrolysis is set at 1 hr, the relative fluorescence at ~1000 relative fluorescence units, and the sensitivity at 780 pg/ml of homologous antigen, then 500 ng/ml of enzyme-antibody conjugate must be used in the ELISA. If, however, the assay has to detect only 3.12 ng/ml of homologous antigen, then the concentration of conjugate can be reduced to 125 ng/ml. It should be noted by comparing the homologous with the heterologous reactions (wells B5 versus B11 and D4 versus D10) that both the specificity and the signal-to-noise ratio for this assay are excellent.

Prepare coating-reagent dilutions

1. Place four 17 × 100–mm test tubes in a rack and add 6 ml PBSN to the last three tubes. In tube 1, prepare a 12-ml solution of coating reagent at 10 μg/ml in PBSN. Transfer 6 ml of tube 1 solution to tube 2. Mix by pipetting up and down five times. Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reagent at 10, 5, 2.5, and 1.25 μg/ml.

2. Using a multichannel pipet, dispense 50 μl of the coating reagent solutions into wells of four Immulon microtiter plates (i.e., each plate is filled with one of the four dilutions). Incubate overnight at room temperature or 2 hr at 37°C.

3. Rinse and block plates with blocking buffer as in steps 5 to 7 of the basic protocol.

Prepare secondary-reagent dilutions

4. Place five 12 × 75–mm test tubes in a rack and add 3 ml blocking buffer to the last four tubes. In tube 1, prepare a 4-ml solution of secondary reagent at 200 ng/ml in PBSN. Transfer 1 ml of tube 1 solution to tube 2. Pipet up and down five times. Repeat this transfer and mix for tubes 3 to 5; the tubes now contain the secondary reactant at 200, 50, 12.5, 3.125, and 0.78 ng/ml. If possible, prepare and test serial dilutions of a nonreactive heterologous form of the secondary reactant in parallel (Fig. 2.1.7).

   If the assay is especially insensitive, it may be necessary to increase the secondary reactant concentrations so the tube-1 solution is 1000 ng/ml.

5. Dispense 50 μl of the secondary reagent solutions into the first five columns of all four coated plates. The most dilute solution is dispensed into column 5, while solutions of increasing concentration are added successively into columns 4, 3, 2, and 1. Thus, the fifth column contains 0.78 ng/ml and the first column 200 ng/ml. Incubate 2 hr at room temperature.

6. Wash plates as in steps 9 to 11 of the basic protocol.

Prepare developing-reagent dilutions

7. Place five 17 × 100–mm test tubes in a rack and add 3 ml blocking buffer to the last four tubes. In tube 1, prepare a 6-ml solution of developing reagent at 500 ng/ml in blocking buffer. Transfer 3 ml of tube 1 solution into tube 2 and mix. Repeat this transfer and mixing for tubes 3 and 4—the tubes now contain the developing reagent at 500, 250, 125, 62.5, and 31.25 ng/ml.

8. Dispense 50 μl of the developing reagent solutions into the wells of rows 2 to 6 of each plate, dispensing the most dilute solution into row 6 and solutions of increasing concentration successively into rows 5, 4, 3, and 2. Incubate 2 hr at room temperature.

9. Wash plates as in steps 9 to 11 of the basic protocol.

Measure hydrolysis

10. Add 75 μl MUP or NPP substrate solution to each well, incubate 1 hr at room temperature, and measure the degree of hydrolysis visually or with a microtiter plate reader. An appropriate assay configuration results in 0.50 absorbance units/hr at 405 nm when using NPP as a substrate or 1000 to 1500 fluorescence units/hr when using MUP as a substrate.

    These results can be used to adjust optimal concentrations in the basic and alternate protocols.

PREPARATION OF ANTIBODY–ALKALINE PHOSPHATASE CONJUGATES

SUPPORT PROTOCOL

Antibodies are mixed with alkaline phosphatase and cross-linked by incubation with glutaraldehyde for 2 hr. The reaction is stopped by adding lysine and ethanolamine contained in PBSLE. The mixture is then desalted on a small Sephadex G-25 sizing column and the fractions are analyzed to detect those containing conjugate.

Additional Materials

>0.2 mg/ml antibody in PBS
Alkaline phosphatase in NaCl solution (Sigma #P0905)
25% glutaraldehyde, EM grade (Sigma #G5882)
PBS containing 100 mM lysine and 100 mM ethanolamine (PBSLE)
Blocking buffer containing 2.5 mM $MgCl_2$
10-ml Sephadex G-25 column (*APPENDIX 3*)
0.2-μm filter

1. Prepare a 1:3 mixture of antibody/alkaline phosphatase in PBS at >0.2 mg/ml total protein concentration.

   Because of the high specific activity and long shelf-life of most antibody–alkaline phosphatase conjugates, an initial preparation of 0.5 mg antibody and 1.5 mg alkaline phosphatase will usually produce enough conjugate to analyze 200 to 800 microtiter plates.

2. Add 25% glutaraldehyde to 0.2% final while vortexing. Incubate 2 hr at room temperature. Stop reaction by adding an equal volume of PBSLE.

3. Desalt the sample by chromatography on a Sephadex G-25 column in PBSN; bed volume of the column should be 5 to 10 times larger than the reaction volume. Collect fractions that are one-half the volume of the reaction volume.

4. Assay fractions by transferring 2 μl into tubes containing 0.5 ml NPP substrate solution. Pool the first five fractions that strongly hydrolyze NPP.

   While it is not essential to remove coupled from uncoupled reactants, this method will enrich for enzyme-antibody conjugates.

5. Mix the pool 1:2 in blocking buffer containing 2.5 mM $MgCl_2$, filter through a 0.2-μm filter, and store at 4°C.

REAGENTS AND SOLUTIONS

Borate-buffered saline (BBS)

0.17 M H_3BO_4
0.12 M NaCl
Adjust to pH 8.5 with NaOH

Blocking buffer

BBS (see above) containing:
0.05% Tween 20
1 mM EDTA
0.25% bovine serum albumin (BSA)
0.05% NaN_3
Store at 4°C

Gelatin may be substituted for BSA; 5% instant milk has been successfully used but may interfere nonspecifically with antibody binding.

MUP substrate solution

0.2 mM 4-methylumbelliferyl phosphate (MUP; Sigma #M8883)
0.05 M $NaCO_3$
0.05 mM $MgCl_2$
Store at room temperature

NPP substrate solution

3 mM *p*-nitrophenyl phosphate (NPP; Sigma #104-0)
0.05 M $NaCO_3$
0.05 mM $MgCl_2$
Store at 4°C

Test antibody solution

Hybridoma supernatants (*UNIT 2.6*) can usually be diluted 1:5 and ascites fluid and antisera (UNIT 2.4) diluted 1:500 in blocking buffer and still generate a strong positive signal. Dilutions of nonimmune ascites or sera should be assayed as a negative control. Prepare antibody dilutions in cone- or round-bottom microtiter plates before adding them to antigen-coated plates.

Sources of appropriate antibodies and conjugates can be found in Linscott's Directory of Immunological and Biological Reagents.

Test antigen solution

0.2 to 10 μg/ml antigen, purified or partially purified in PBSN; store at 4°C

Wash buffer

Hanks balanced salt solution (HBSS; *APPENDIX 2*)
1% fetal calf serum (FCS; heat-inactivated 60 min, 56°C)
0.05% NaN_3
Store at 4°C

COMMENTARY

Background Information

Since their first description in 1971 (Engvall and Perlman), ELISAs have become the system of choice when assaying soluble antigens and antibodies. Factors that have contributed to their success include their sensitivity, the long shelf-life of the reagents (alkaline phosphatase conjugates typically lose only 5% to 10% of their activity per year), the lack of radiation hazards, the ease of preparation of the reagents, the speed and reproducibility of the assays, and the variety of ELISA formats that can be generated with a few well-chosen reagents. Additionally, no sophisticated equipment is necessary for many ELISA applications, including screening hybridoma supernatants for specific antibodies and screening biological fluids for antigen content.

The ELISAs described here combine the special properties of antigen-antibody interactions with simple phase separations to produce powerful assays for detecting biological molecules. The multivalency of antibodies can result in the formation of long-lived antigen-antibody complexes, thus allowing long periods of time during which such complexes can be measured. By designing an assay so that a capture reagent initiates the binding of antigen-antibody complexes and enzyme conjugates onto a solid phase, the unbound reagents can be easily and rapidly separated from the solid phase. The solid phase is washed and the amount of bound conjugate is visualized by incubating the solid phase with a substrate that forms a detectable product when hydrolyzed by the bound enzyme. ELISAs are similar in principle to radioimmunoassays, except that the radioactive label is replaced by an enzyme conjugate.

A number of different enzymes have been successfully used in ELISAs, including alkaline phosphatase, horseradish peroxidase, β-galactosidase, glucoamylase, and urease. Alkaline phosphatase—perhaps the most widely used conjugated enzyme—is recommended because of its rapid catalytic rate, excellent intrinsic stability, availability, ease of conjugation, and resistance to inactivation by common laboratory reagents. Additionally, the substrates of alkaline phosphatase are nontoxic

Double-Immunodiffusion Assay for Detecting Specific Antibodies

BASIC PROTOCOL

Double immunodiffusion is a simple gel-based assay for detecting antigen-specific antibodies. Analytical agar gels are poured onto microscope slides that have been precoated with agar. Small wells are punched 0.5- to 0.75-cm apart in the analytical gel. Antigen and antibody solutions are placed in adjacent wells and allowed to diffuse into the gel for 6 to 48 hr. As antibody and antigen form diffusion gradients that cross each other, a line of immunoprecipitation may form between the wells, indicating the presence of specific antibodies. The gel is then stained and destained until precipitin lines are maximally visible.

Materials

Noble agar (Difco)
PBS (*APPENDIX 2*) containing 0.05% NaN_3 (PBSN)
4% PEG 6000 (J.T. Baker) in PBSN, prewarmed to 56°C (store at room temperature)
1 mg/ml antigen
Antisera
Staining solution
Destaining solution

2 × 3–in. microscope slides, precleaned
Boiling and 56°C water baths
50°C oven
Template (see Fig. 2.3.1)
15-G stainless steel needle (blunt-ended and beveled) *or* immunodiffusion punch set (EC Apparatus)
10-µl Hamilton syringe
Humidified chamber (enclosed plastic container with moistened tissues; Fig. 3.8.1)
Staining rack and dish
Whatmann 3MM filter paper

Precoat microscope slides with agar

1. Prepare a 0.5% noble agar solution in PBSN and place in a boiling water bath until agar dissolves.
2. Place 2 × 3–in. microscope slides on a level surface and pipet 8 ml of 0.5% melted agar evenly over the surface of each slide. Do not disturb gels until the agar has set.
3. Allow gels to dry 4 hr in a 50°C oven or overnight at room temperature.

   A dried agar precoat provides an adhesive base that prevents the analytical agar from separating from the slide during staining and destaining treatments.

Prepare analytical gel

4. Dissolve 2% noble agar in PBSN in a boiling water bath.
5. Place coated microscope slides on a level surface. Cool the 2% melted agar to 56°C. Mix 2% melted agar 1:1 with 56°C PEG solution and pipet 10 ml evenly over each slide. Do not disturb gels until the agar has set.

   PEG stabilizes immunoprecipitates and increases their visibility.

6. Place the agar gel over a template and, using a blunt-ended and beveled 15-G needle or an immunodiffusion punch set, carefully punch wells to accommodate all antigen and antisera solutions to be tested (see Fig. 2.3.1).

7. Remove agar plugs using a Pasteur pipet attached to a vacuum line.

   Use a weak vacuum to remove the agar plugs, taking care not to disturb the surrounding agar field.

Load the gel

8. Prepare three antigen samples to be tested against undiluted antisera—one should be ~1 mg/ml, and the other two should be ~500 and 250 µg/ml (prepared by diluting 1 mg/ml antigen 1:1 serially with PBSN. Using a 10-µl Hamilton syringe or pipettor, fill the central wells with an antigen sample and surrounding wells with antisera (the wells hold 5 to 10 µl). Maintain slides on a level surface, and allow samples to diffuse into the gel.

   When screening antisera, multiple dilutions of antigen should be tested against all antisera. To increase the amount of reagent loaded, wells can be filled 2 or 3 times. After the liquid is absorbed into the gel (~5 to 10 min), the wells may be refilled with antigen solution.

9. Place loaded gels in a humidified chamber and incubate 48 hr at room temperature. Examine the gels and score for precipitin lines at 6, 24, and 48 hr.

   Gels should not be in direct contact with the moistened tissues in the humidity chamber.

Wash and stain the gels

10. Place gels in a staining rack. Place rack in a staining dish filled with PBSN and incubate 24 hr at room temperature with gentle stirring using a magnetic stirbar.

    Washing is done in steps 10 to 12 to remove proteins that are not precipitated.

11. Replace PBSN with fresh solution and incubate 24 hr at room temperature.

12. Remove salt by replacing PBSN with water. Incubate 4 hr at room temperature.

13. Remove gels from the staining rack and place face-up on a flat surface. Dry the gels by covering with 3MM filter paper and leaving overnight at room temperature.

14. Place dry gels in a staining rack and immerse 10 min in staining solution at room temperature.

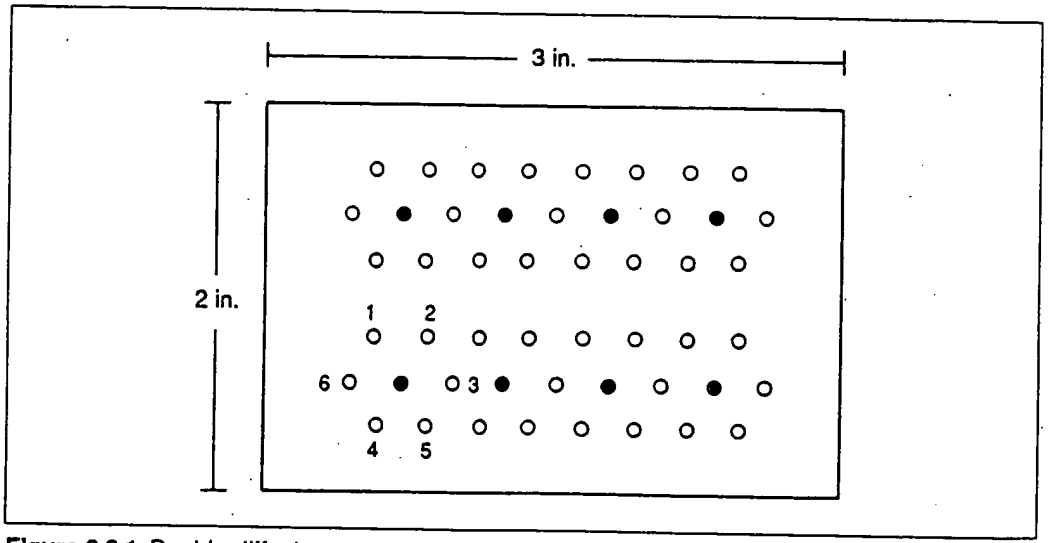

Figure 2.3.1 Double-diffusion template consisting of eight partially overlapping hexagonal arrays distributed around eight central wells. Central wells are represented by dark circles. A numbered set of wells arranged hexagonally around a central well is shown.

15. Destain by immersing gels 4 min in destaining solution. Repeat until precipitin lines are maximally visible and the background staining is negligible.

16. Air dry the gels at room temperature.

REAGENTS AND SOLUTIONS

Destaining solution

15% (vol/vol) ethanol
5% (vol/vol) glacial acetic acid
80% H_2O
Store at room temperature

Staining solution

0.5% (wt/vol) Coomassie Brilliant Blue R-250
40% (vol/vol) ethanol
10% (vol/vol) glacial acetic acid
50% H_2O
Store at room temperature

COMMENTARY

Background Information

Gel-diffusion techniques, among the earliest methods for detecting specific antibodies and for measuring antigenicity (Ouchterlony and Nilsson, 1986), are still useful methods for detecting specific antibodies. However, they do require high concentrations of both antigen and antibody and are relatively insensitive to antibodies with low affinities. Recently, a silver-staining technique has been described that increases the sensitivity of double-diffusion assays 10 to 100 times (Rochu et al., 1989).

Double immunodiffusion owes its success to the unique nature of antibody-antigen interactions. When polyvalent antibodies with moderate-to-high intrinsic affinities are mixed with antigen at the right ratio—called the zone of equivalence—lattices of antibody-antigen complexes form and precipitate out of solution. When gradients of antigen and antibody are established by diffusion from adjacent wells in a bed of agar, a line of practically insoluble precipitation forms at the equivalence zone (precipitin lines).

Critical Parameters

In this assay the initial antigen and antibody concentrations must be able to support the formation of equivalence zones. For this reason, three different antigen concentrations are recommended. If no lines of precipitation are observed, more sensitive techniques, (e.g., ELISAs) should be considered.

Anticipated Results

Double-diffusion assays in which the immunoprecipitates are stained with Coomassie Brilliant Blue can be sensitive to as little as 25 µg/ml of specific antibody. In the absence of staining, the assay is sensitive to ~100 µg/ml of specific antibody. Details of the precipitation patterns (double precipitin lines, spurs, and lines of identity) can reveal information about the antigenic specificities of various antisera and information about the structure of the antigen (Ouchterlony and Nilsson, 1986). Figure 2.3.2 illustrates typical results.

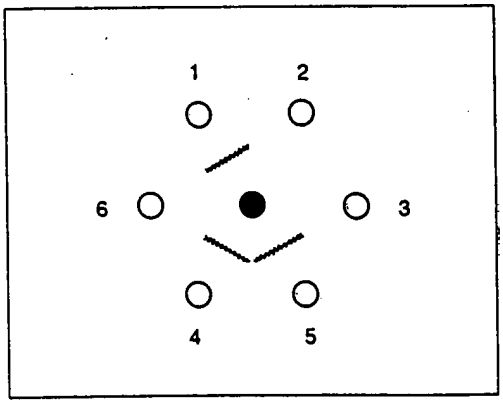

Figure 2.3.2 Typical results of a double-immunodiffusion assay. Wells 1, 4, and 5 are positive for reactive antisera, while wells 2, 3, and 6 are negative.

Time Considerations

The higher the titer of specific antibody, the more rapid the precipitation. Precipitation may begin in some systems within a few hours, while in others it may take 24 to 48 hr to complete.

Literature Cited

Ouchterlony, O. and Nilsson, L-A. 1986. Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology, Vol. 1: Immunochemistry (D.M. Weir, L.A. Herzenberg, C. Blackwell, and L.A. Herzenberg, eds.) pp. 32.1-32.50. Blackwell, Oxford.

Rochu, D., Crespeau, H., Fine, A., and Fine, J-M. 1989. A sensitive double-diffusion microassay suitable for the detection of idiotype–anti-idiotype precipitates. *J. Immunol. Methods* 118:67-71.

Key Reference

Ouchterlony and Nilsson, 1986. See above.

Contains a detailed description of immunodiffusion techniques and provides detailed interpretations of various patterns of immunoprecipitation observed in double-immunodiffusion experiments.

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